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(54) Title: BRASSICACEAE WITH ALTERED FLORAL DEVELOPMENT

(57) Abstract: Provided are means and methods for obtaining plants from the family Brassicaceae, particularly plants from the genus Brassica and closely related genera, with altered floral development by providing plants with a chimeric gene, characterized in that upon introduction into the genome of a plant of the family Brassicaceae of the chimeric gene the phenotypic expression of a target B-type floral organ identity gene is reduced selectively in whorl 2.

Brassicaceae with altered floral development.

Field of the invention

The present invention relates to means and methods for obtaining plants from the family *Brassicaceae*, particularly plants from the genus *Brassica* and closely related genera, with altered floral development. More particularly the present invention relates to means and methods for genetically modifying *Brassicaceae* plants in such a way that resulting transgenic plants exhibit altered flowers wherein only one whorl of floral organs is affected. The invention also relates to *Brassicaceae* plants having two whorls of sepals or sepaloids and no whorl of petals while displaying a developmentally normal whorl of stamens.

Background

The agricultural interest in *Brassicaceae* plants, particularly *Brassicaceae* plants grown for oil production, such as oilseed rape, which develop flowers without petals, has been spurred by the observation that senescent petals from oilseed rape plants falling on and around the plants are ideal nursery sites for the development of spores of certain fungi, such as e.g. *Sclerotinia sclerotiorum*. This fungus is responsible for significant damages in oilseed rape cultures (Lamarque, 1983). By depriving the sclerotinia spores from their initial food source, the risk of disease development is greatly reduced.

In addition, the floral canopy of oilseed rape compromises the yield potential of the crop as the transmission of the photosynthetically active radiation to the leaf canopy is significantly reduced during anthesis. Studies have determined that the petals absorb 64% and reflect 14% of the photosynthetically active radiation (Fray et al., 1996; Rao et al. 1991).

Brassicaceae plants having flowers without petals ("apetalous *Brassicaceae* plants") have been described in the art.

The apetalous character of *Brassica napus* variant N-o-112 is controlled by two major genetic loci and a third modifying locus. These three loci have been introgressed into a commercial cultivar using marker assisted backcrossing (Robinson et al., 1999)

HyLite201 is a commercial apetalous canola variety reported to have a significant reduced incidence of *Sclerotinia* infection and disease symptom development when compared to untreated conventional varieties.

The published PCT patent application "WO" 99/15679 describes a petal-specific promoter and a method for obtaining plants without petals by introducing into the plants a chimeric gene comprising a cytotoxine encoding open reading frame under the control of the petal-specific promoter.

WO 99/64599 describes expression of the Cyclin dependent kinase inhibitor ICK1 coding sequence under control of the APETALA3 promoter to produce Arabidopsis plants with modified petals and/or with male sterility.

Two major problems, both resulting in a poor pollination and consequently lower seed yield, are associated with *Brassicaceae* plants or *Brassica* plants having flowers without petals. On the one hand, the absence of one whorl of floral organs results in more fragile flowers, which easily fall apart. On the other hand, the absence of the petals results in "holes" between the sepals since sepals and petals are placed alternately in a wild type *Brassicaceae* or *Brassica* flower. Insects can easily reach the nectaries through this holes rather than reaching the nectaries from the top of the flower and consequently less pollen is spread by insects feeding on the apetalous flowers.

Brassicaceae plants having flowers without petals, but wherein the petals have been replaced by other floral organs, have also been described in the art.

In *Arabidopsis thaliana*, homeotic mutations were discovered that result in the development of abnormal flowers in which one type of flower part is replaced by another. These mutations identify the floral organ identity genes. The homeotic floral mutations in Arabidopsis fall into three classes, each of which affects the organs of two adjacent whorls.

The mutant phenotypes can be explained using the so-called ABC model of gene activity of the floral organ identity genes. Briefly, the ABC model can be explained as follows.

A flower is composed of four concentric whorls of structures which reflect the arrangement of the floral organ primordia in the floral meristem. The sepals (whorl1) form from the outermost ring of meristem tissue and the petals (whorl 2) form from a ring of tissue lying immediately inside it. An inner ring of tissue gives rise to the stamens (whorl 3). The female reproductive organs –the carpels (whorl 4)- develop from the center of the meristem.

The floral meristem can also be divided into three overlapping regions A, B and C, whereby region A covers whorls 1 and 2; region B covers whorls 2 and 3 and C covers whorls 3 and 4. Three regulatory functions –*a*, *b* and *c*- function in the regions A, B and C and combinatorially give each whorl a unique identity. In addition *a* function inhibits *c* function in whorls 1 and 2 and *c* function inhibits *a* function in whorls 3 and 4. Function *a* corresponds to the activity of *a* function genes such as *APETALA2* and *APETALA1* , *b* to *APETALA3* and *PISTILLATA*, and *c* to *AGAMOUS*. Sepals require *APETALA1* and *APETALA2* (*APETALA2* is expressed in all whorls, but acts in organ identity specification only along with the *APETALA1* gene whose expression is limited to whorl 1 and 2); petals require a combination of *APETALA1* and *APETALA2* together with *APETALA*3 and *PISTILLATA*; stamens require *APETALA*3 and *PISTILLATA* with *AGAMOUS*; and carpels require *AGAMOUS* alone. Mutations that alter the pattern of

expression of one or more of these genes result in differing patterns of expression of the others and homeotic transformations of the floral parts.

The following homeotic flower mutants have flowers without petals:

apetala2 :The floral phenotype of the *ap2* allelic series may be summarized as follows: the first whorl organs from leaf-like to carpels ; second whorl organs may vary from staminoid organs (weak ap2-1 allele) to failure of the organs to develop (strong ap2-2 allele).

apetala3 or *pistillata* mutant: has flowers with the following whorls: sepal, sepal, carpel, carpel.

apetala3/pistillata double mutant: has flowers with the following whorls: sepal, sepal,carpel, carpel.

apetala2/apetala3 double mutant: has flowers with the following whorls: double mutant carpel, carpel, carpel, carpel.

In the absence of all three classes of genes, the flower consists of whorls of identical leaf-like organs. Addition of *a* function genes to the ground state results in all sepals, while addition of *c* function to the ground state gives all carpels. Addition of both *a* and *c* functions results in flowers with half-flower phenotype sepal, sepal carpel, carpel.

Recently, (Pelaz et al, 2000) it has been shown that B and C floral identity functions require MADS-box genes *SEPALLATA1/2/3* for their proper functioning. Honma and Goto (2001) demonstrated that the class B proteins of Arabidopsis, *PISTILLATA* and *APETALA3*, interact with *APETALA1* and *AGAMOUS* through *SEPALLATA3*.

WO93/21322 describes a method for controlling the morphogenesis of plant organs which comprises causing the underexpression of a homeotic gene responsible for determining the identity of the plant organ in object. Particularly, petunia flowers are converted to define petals with sepaloid characters by

introducing a transgene construct corresponding to the *green petal* gene of this application under control of a promoter that shows expression in the second whorl of flowers.

WO 94/00582 describes a method for obtaining plants with altered flower morphology characterized in that the plants have a recombinant polynucleotide integrated into their genome, essentially comprising an inhibitory gene which upon proper expression in the floral meristem cells is capable of inhibiting the development and determination of floral primordia into petal and/or stamen primordia. In a preferred embodiment, the inhibitory gene is an antisense or sense/co-suppression gene directed against a MADS box target gene, particularly target genes encoding the fbp1 or fbp2 proteins as disclosed therein or proteins which are homologous therewith. In another embodiment, the promoter of the recombinant polynucleotide comprises a MADS box gene promoter, preferably of the B-type.

Although the above mentioned plants with homeotic mutations would not necessarily suffer from the problems mentioned above in connection to apetalous *Brassicaceae* plants, other problems are associated with the use of these plants. In particular, the spontaneous homeotic mutations are recessive alleles, thereby complicating the maintenance of the phenotype, particularly in hybrid *Brassicaceae* plants.

Moreover, inactivation of the floral organ identity genes as described, either by mutagenesis or using a transgenic approach, to replace petals by sepals or sepaloids, affects more than one whorl of floral organs, resulting in reproductive problems, particularly male-sterility.

The prior art is thus deficient in providing methods and means for obtaining *Brassicaceae* plants which have a dominant allele, the expression of which results in firm, closed flowers without petals, without affecting floral organs of

another whorl, particularly without affecting the reproductive organs of the flowers.

The current invention has solved these and other problems as set forth hereinafter in the various embodiments, as well as in the claims.

Summary of the invention

The invention provides a method for obtaining a plant from the family *Brassicaceae*, such as but not limited to a plant from the genus *Brassica* or a closely related genus, or oilseed rape, with altered floral morphology comprising the steps of introducing into a cell of that plant a B-type floral organ identity specific chimeric gene to generate a transgenic cell; and regenerating a transgenic plant from that transgenic cell, characterized in that the B-type floral organ identity specific chimeric gene is capable of reducing or inhibiting the phenotypic expression of a target B-type floral organ identity gene selectively, or exclusively in the whorl 2 of a flower or flower primordia of transgenic plants and that the flowers of the transgenic plant have an additional whorl of sepals or sepaloids; no whorl of petals; and a whorl of functional stamens when compared to flowers of normal untransformed plants, or characterized in that the B-type floral organ identity specific chimeric gene comprises a promoter of an A-type floral organ identity gene, or a promoter from *APETALA1* or a gene homologous to *APETALA1*, such as but not limited toa promoter of an A-type floral organ identity gene comprising the nucleotide sequence of SEQ ID No 3 or SEQ ID No 4. Alternatively, the B-type floral organ identity specific chimeric gene comprises comprises a promoter directing transcription of selectively, preferably exclusively in whorl 2 of flowers or flower primordia when introduced into cells of plants of the genus *Brassica*, such as but not limited to a modified promoter from the *APETALA 3* gene from *Arabidopsis thaliana*, or a promoter comprising the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 1 to the nucleotide at position 152 and the nucleotide sequence of SEQ ID No 5 from the

nucleotide at position 462 to the nucleotide position at position 751 with the provisio that it does not comprise the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 198 to the nucleotide at position 461.

In one embodiment of the invention the B-type floral organ identity specific gene comprises a first nucleotide sequence having at least 70% sequence identity to a part of at least 25 consecutive nucleotides of the nucleotide sequence of a target B-type floral organ identity gene of the target plant excluding the sequence encoding the MADS box, which may be selected from the group of *APETALA3*, *PISTILLATA*, a gene homologous to *APETALA3* and a gene homologous to *PISTILLATA*; or comprising a first nucleotide sequence having at least 70% sequence identity to the complement of a part of at least 25 consecutive nucleotides of the nucleotide sequence of a target B-type floral organ identity gene of that plant.

In another embodiment of the invention, the B-type floral organ identity specific gene comprises a first nucleotide sequence having at least 70% sequence identity to a nucleotide sequence of at least 25 consecutive nucleotides selected from the group consisting of the nucleotide sequence of SEQ ID No 1, the nucleotide sequence of SEQ ID No 2, the complement of the nucleotide sequence of SEQ ID 1 and the complement of the nucleotide sequence of SEQ ID 2. Optionally, the B-type floral organ identity specific chimeric gene comprises a second nucleotide sequence having at least 70% sequence identity to a part of at least 25 consecutive nucleotides of the nucleotide sequence of a target B-type floral organ identity gene of the plant or complementary to that nucleotide sequence of the target B-type floral organ identity gene of the plant (excluding the sequence encoding the MADS box), and wherein the RNA molecule generated upon transcription of the B-type floral organ identity gene-silencing chimeric gene is capable of forming a double-stranded region between the regions of the transcribed RNA molecule transcribed from the first and second

nucleotide sequence. Optionally, a spacer region, which may comprise an intron, is comprised between the first and second nucleotide sequence.

1. In another embodiment, the invention provides a DNA molecule comprising a B-type floral organ identity specific chimeric gene, characterized in that upon introduction into the genome of a plant of the family *Brassicaceae* of the B-type floral organ identity specific chimeric gene, that B-type floral organ identity specific chimeric gene is capable of reducing or inhibiting the phenotypic expression of a target B-type floral organ identity gene selectively in whorl 2 of a flower or flower primordia and that flowers of that transgenic plant have an additional whorl of sepals or sepaloids; no whorl of petals; and a functional whorl of stamens when compared to flowers of normal untransformed plants, or characterized in that the B-type floral organ identity specific chimeric gene comprises a promoter of an A-type floral organ identity gene, such as a promoter from *APETALA1* or a gene homologous to *APETALA1*, which may comprise the nucleotide sequence of SEQ ID No 3 or SEQ ID No 4. Alternatively, the B-type floral organ identity specific chimeric gene comprises a promoter directing transcription of selectively, or exclusively in whorl 2 of flowers or flower primordia when introduced into cells of plants of the genus *Brassica*, such as but not limited to a modified promoter from the APETALA 3 gene from *Arabidopsis thaliana*, comprising the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 1 to the nucleotide at position 152 and the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 462 to the nucleotide position at position 751 with the provisio that it does not comprise the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 198 to the nucleotide at position 461.

In yet another embodiment of the invention, a plant from the family *Brassicaceae*, such as a plant from the genus *Brassica* or a closely related genus, or oilseed rape, comprising a DNA molecule having a B-type floral organ identity specific chimeric gene according to invention integrated into the genome of the cells of

that plant is provided as well as seeds including crushed seed of that plants which comprise the chimeric genes of the invention.

Also provided by the invention is the use of a DNA molecule having a B-type floral organ identity specific chimeric gene according to invention to obtain a plant of the family *Brassicaceae* with altered flowers having an additional whorl of sepals or sepaloids; no whorl of petals; and a functional whorl of stamens when compared to flowers of normal untransformed plants.

Detailed description of preferred embodiments

The current invention is based on the observation that the introduction into a plant of the *Brassicaeae* family, such as oilseed rape or *Arabidopsis* plants, of a chimeric gene capable of reducing the expression of an endogenous B-type floral organ identity gene selectively in whorl 2, i.e. the whorl which would normally develop into petals in non-transgenic plants results in transgenic plants having flowers wherein the petals have a sepaloid character or are converted into an additional whorl of sepals, without affecting the development of functional stamens. These transgenic plants thus posses a dominant allele which when expressed results in compact flower buds with two rows of sepals or sepaloids and no petals but otherwise normal flowers.

In one embodiment of the invention, a method is provided for obtaining a plant from the family *Brassicaceae* with altered floral morphology comprising the following steps:

a. introducing a chimeric gene capable of reducing or inhibiting, selectively in the whorl of petals, the phenotypic expression of a floral organ identity gene of the B-type (a "B-type floral organ identity specific chimeric gene") into a cell of the plant to generate a transgenic cell; and
b. regenerating a transgenic plant from said transgenic cell.

Flowers of said transgenic plant have an additional whorl of sepals or a whorl of petals with sepaloid character, no whorl of petals; and a whorl of functional stamens, when compared to flowers of normal untransformed plants.

As used herein "a plant with altered floral morphology", or "a double sepaloid plant" refers to a plant wherein the petals of the flower exhibit a sepaloid character (e.g. wherein petals are greener and/or shorter than in wild type plants), or wherein the whorl of petals has been replaced by a whorl of sepals. In other words, flowers have an additional whorl of sepaloids or sepals. The double sepaloid flowers according to the invention have a functional whorl of stamen, i.e.

are capable of producing a sufficient amount of pollen to cross-pollinate another plant. Preferably, the stamen of the altered flowers are morphologically indistinguishable from stamen of wild type flowers. "Sepaloid" can also be used to indicate petals with sepaloid character as described herein.

As used herein, a "B-type floral organ identity specific chimeric gene" is a chimeric gene which, when introduced into a plant of the family of the *Brassicaceae*, such as but not limited to a plant of the genera *Brassica, Arabidopsis* or a closely related genus, is capable of reducing or inhibiting selectively or exclusively, in the whorl of petals (whorl 2) the phenotypic expression of a floral organ identity gene having a *b* function as described above in the ABC model, such as the *Arabidopsis* genes *APETALA3* and *PISTILLATA* or the homologous genes from plants of the genus *Brassica*, such as the genes isolated from *Brassica napus* homologous to *APETALA3* and *PISTILLATA* which comprise the nucleotide sequences of SEQ ID No 1 or 2, respectively.

For the purpose of this invention a "B-type floral organ identity gene" is a floral organ identity gene comprising a MADS box, having a *b* function in the ABC model, such as the *Arabidopsis* genes *APETALA3* and *PISTILLATA* or the homologous genes from plants of the genus *Brassica*, such as the genes isolated from *Brassica napus* homologous to *APETALA3* and *PISTILLATA* which comprise the nucleotide sequences of SEQ ID No 1 or 2, respectively.

"Selective reducing or inhibiting of the phenotypic expression" of a floral identiy gene having a b function as used in the context of this invention indicates that the reduction of the phenotypic expression of the B-type floral organ identity gene (and concurrent conversion of petals into sepals) occurs predominantly in whorl 2 resulting in conversion of petals into sepaloids or sepals, while possible reduction of phenotypic expression of the target B-type floral organ identity gene in other whorls, or even in other parts of the transgenic plant, results only in small or no phenotypic differences, which are irrelevant a practical level. The phenotypic

expression may however be reduced or inhibited exclusively in whorl 2 of a flower.

It goes without saying that "reducing or inhibiting" does not necessarily mean that the phenotypic expression of the target floral organ identity gene is abolished for a 100%. Rather, "reducing or inhibiting" relates to a level of phenotypic expression which is lower than the level of phenotypic expression of the target B-type floral organ identity gene in the whorl of untransformed control plants. Different levels of reduction or inhibition of the phenotypic expression of a B-type floral organ identity gene may be reflected in the degree of conversion of the petal into a sepaloid or sepal, as indicated above.

It will also be clear that within a population of different transformed plants, representing a number of independent transformation events, such different degrees of conversion may be represented. However, the person skilled in the art, would be perfectly able to select the transformed plants or plant lines with the desired phenotypes.

The phenotypic expression of a B-type floral organ identity gene of a plant from the family *Brassicaceae* may conveniently be reduced or inhibited using antisense RNA encoding chimeric genes and/or sense RNA encoding chimeric genes (cosuppresion).

In one embodiment of the invention, the B-type floral organ identity specific chimeric genes comprise a first nucleotide sequence having at least about 70% sequence identity to a part of at least about 25 consecutive nucleotides ("nt") of the nucleotide sequence of the B-type floral organ identity gene present in the plants ("target B-type floral organ identity gene") or to the complement of that part of the sequence. However, longer parts, comprising at least about 50 nt, or at least about 100 nt, or at least about 250 nt, or at least about 500 nt, or longer can equally be used. Similarly, it goes without saying that the first nucleotide

sequence may have higher sequence identity to the mentioned part of the target B-type floral organ identity gene, such as at least about 80% sequence identity, or at least about 90% sequence identity, or about 100% sequence identity, or be identical to the mentioned part of the target B-type floral organ identity gene or the complement thereof.

Nevertheless, in view of the presence of conserved nucleotide sequences encoding the conserved MADS boxes in B-type floral organ identity genes, it is preferred that the nucleotide sequences of the target B-type floral organ identity genes do not comprise the nucleotide sequences encoding the MADS box.

The terms "MADS box" is used within its normal meaning in the art, i.e. the MADS box refers to the conserved domain of about 56 amino acid residues found in a number of transcription factors and which has the following consensus pattern: R-x-[RK]-x(5)-I-x-[DNGSK]-x(3)-[KR]-x(2)-T-[FY]-x-[RK](3)-x(2)-[LIVM]-x-K(2)-A-x-E-[LIVM]-[STA]-x-L-x(4)-[LIVM]-x-[LIVM](3)-x(6)-[LIVMF]-x(2)-[FY] (Wisconsin Package Version 10.1 Genetics Computer Group, Madison, Wisconsin).

When the nucleotide sequence of the target B-type floral organ identity gene endogenous to the *Brassicaceae* plant of interest, or a substantial part thereof, is known, the design of the B-type floral organ identity specific chimeric genes according to the above mentioned scheme is straightforward. Nucleotide sequences known in the art, of *APETALA3*-like genes have been identified from the following non-limiting list of *Brassicaceae* plant species and are indicated by their database accession number between brackets: *Brassica napus* (AF124814); *Brassica rapa* (AF056542); *Brassica rapa* (AF056538); *Brassica oleracea* (AF056537); *Brassica oleracea* (AF056536); *Brassica napus* (AF056535); *Brassica napus* (AF056534); *Brassica napus* (AF056533); *Brassica napus* (AF056532); *Brassica oleracea* (AF034610); *Brassica rapa* (AF056542); *A. thaliana* (AF115814); *A. thaliana* (AF115813); *A. thaliana* (AF115812); *A.*

thaliana (AF115811); *A. thaliana* (AF115810); *A. thaliana* (AF115809); *A. thaliana* (AF115808); *A. thaliana* (AF115807); *A. thaliana* (AF115806); *A. thaliana* (AF115805); *A. thaliana* (AF115804); *A. thaliana* (AF115803); *A. thaliana* (AF115802); *A. thaliana* (AF115801); *A. thaliana* (AF115800); *A. thaliana* (AF115799); *A. thaliana* (AF115798); *A. thaliana* (D21125); *A. thaliana* (AL132971); *A. lyrata* (AF143380); *A. thaliana* (AF056541); *A. thaliana* (M86357). Nucleotide sequences known in the art, of *PISTILLATA*-like genes have been identified from the following non-limiting list of Brassicaceae plant species and are indicated by their database accession number between brackets: A. lyriata (AF143382); A. thaliana (AF198055); A. thaliana (AF115830); A. thaliana (AF115829); A. thaliana (AF115828); A. thaliana (AF115827); A. thaliana (AF115826); A. thaliana (AF115825); A. thaliana (AF115824); A. thaliana (AF115823); A. thaliana (AF115822); A. thaliana (AF115821); A. thaliana (AF115820); A. thaliana (AF115819); A. thaliana (AF115818); A. thaliana (AF115817); A. thaliana (AF115816); A. thaliana (AF115815); A. thaliana (D30807).

When the nucleotide sequence of a target B-type floral organ identity gene endogenous to the *Brassicaceae* plant of interest in unknown, the isolation of a substantial part of the target B-type gene and determination of the nucleotide sequence thereof is well with the reach of the skilled artisan. Substantial parts of a target B-type floral organ identity gene may be identified and isolated e.g. by hybridization of DNA of the *Brassicaceae* plant of interest under stringent conditions (see Sambrook et al.) using as probe a part of at least 25 consecutive nucleotides of a B-type floral organ identity gene (preferably from a part not encoding the MADS box), which has been identified previously and the nucleotide sequence of which may be known (such as but not limited to the nucleotide sequences indicated above, in addition to the nucleotide sequences of *APETALA3*-like genes identified from the following non-Brassicaceae species *Peperomia hirta* (AF052879); *Liriodendron tulipifera* (AF052878); *Michelia figo* (AF052877); *Ranunculus bulbosus* (AF052876); *Dicentria eximia* (AF052875);

Papaver nudicaule (AF052874); *Papaver nudicaule* (AF052873); *Papaver cliformicum* (AF052872); *Pachysandra* (AF052871); *Pachysandra terminalis* (AF052870); *Syringa vulgaris* (AF052869); *Lycopersicon esculentum* (AF052868); *Oryza sativa* (AF077760); *Ranunculus ficaria* (AF130870); *Ranunculus bulbosus* (AF130869); *Sanguinaria canadensis* (AF130868); *Papaver nudicaule* (AF052855); *Ranunculus ficaria* (AF052854) *Oryza sativa* (AF077760) or the nucleotide sequences of *PISTILLATA*-like genes identified from the following non-Brassicaceae species: *Hyacintus orientalis* (AF134115); *Hyacintus orientalis* (AF134114); *Ranunculus ficaria* (AF130872); *Sanguinara canadensis* (AF130871); *Piper magnificum* (AF052867); *Piper magnificum* (AF052866); *Peperomia hirta* (AF052865); *Lirodendron tulipifera* (AF052864); *Michelia figo* (AF052863); *Delphinium ajacis* (AF052862); *Syringa vulgaris* (AF052861); *Ranunculus bulbosus* (AF052860); *Ranunculus bulbosus* (AF052859); *Ranunculus ficaria* (AF052858); *Dicentra eximia* (AF052857); *Papaver nudicaule* (AF052856); *Papaver nudicaule* (AF052855); *Ranunculus ficaria* (AF052854)). Subsequently, the identified DNA may be isolated and or cloned. The DNA to be hybridized may be cDNA derived from total RNA isolated from flower buds.

Substantial parts of a target B-type floral organ identity genes may also be identified using DNA amplification methods, including PCR, using appropriate oligonucleotides, such as the oligonucleotide sequences having the nucleotide sequence of SEQ ID 6, 7, 8 or 9, as primers. Again, template DNA may be cDNA derived from total RNA isolated from flower buds.

For the purpose of this invention, the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e. a position in an alignment where a residue is present in one sequence but not in the other is regarded as a position with non-identical residues. The alignment of

the two sequences is performed by the Needleman and Wunsch algorithm (Needleman and Wunsch 1970) Computer-assisted sequence alignment above, can be conveniently performed using standard software program such as GAP which is part of the Wisconsin Package Version 10.1 (Genetics Computer Group, Madision, Wisconsin, USA) using the default scoring matrix with a gap creation penalty of 50 and a gap extension penalty of 3.

For the purpose of the invention, the "complement of a nucleotide sequence represented in SEQ ID No: X" is the nucleotide sequence which would be capable of forming a double stranded DNA molecule with the represented nucleotide sequence, and which can be derived from the represented nucleotide sequence by replacing the nucleotides through their complementary nucleotide according to Chargaff's rules (A⇔T; G⇔C) and reading in the 5' to 3' direction, i.e in opposite direction of the represented nucleotide sequence.

In one embodiment of the invention, B-type floral organ identity specific chimeric genes, suitable for use in Arabidopsis or oilseed rape plants, comprise a nucleotide sequence having at least 70% sequence identity to a part of at least about 25 consecutive nucleotides of the nucleotide sequence of SEQ ID No: 1, or its complement, or the nucleotide sequence of SEQ ID No: 2, or its complement. Again, longer parts, comprising at least about 50 nt, or at least about 100 nt, or at least about 250 nt, or at least about 500 nt, up to the complete nucleotide sequences of SEQ ID No 1, SEQ ID No 2 or their complements, can equally be used. The nucleotide sequence may have higher sequence identity to the mentioned nucleotide sequences of SEQ ID No1, SEQ ID No 2 or their complements. E.g. the sequence may have at least about 80% sequence identity, or at least about 90% sequence identity, or about 100% sequence identity, quite particularly be identical to the mentioned sequences.

In another embodiment of the invention, B-type floral organ identity specific chimeric genes comprise both a sense and antisense region, which may be separated by a region of unrelated nucleotide sequences (hereinafter "a spacer"),

and which are capable of forming a hairpin RNA molecule as described in WO 99/93050, wherein the parts of the RNA transcribed from the sense and antisense nucleotide sequences are essentially complementary to each other and capable of forming a artificial hairpin structure with a double stranded RNA region. As also described in WO 99/93050, the efficiency of gene silencing and thus of reduction of phenotypic expression of the *b*-function may be further improved by including an intron in the spacer region. B-type floral organ identity specific chimeric genes of this type may thus comprise a first nucleotide sequence and a second nucleotide sequence wherein the first and second nucleotide sequences are according to the embodiments mentioned above, and the RNA molecule parts transcribed from said first and second nucleotide sequence are capable of forming a double stranded RNA molecule. Several B-type floral organ identity specific chimeric genes of this type including a first nucleotide sequence comprising the nucleotide sequence of SEQ ID No 1 and a second nucleotide sequence comprising the complement of the nucleotide sequence of SEQ ID No 1 or including a first nucleotide sequence comprising the nucleotide sequence of SEQ ID No 2 and a second nucleotide sequence comprising the complement of the nucleotide sequence of SEQ ID No 2, are also described in the Examples section hereinafter.

The reduction of the phenotypic expression of a floral organ identity gene selectively in the whorl of the petals or the primordia thereof can conveniently be achieved by incorporating into the B-type floral organ identity specific chimeric gene a promoter of a floral organ identity gene of an adjacent whorl of flower structures, such as but not limited to a promoter of an A-type floral organ identity gene.

As used herein, an "A-type floral organ identity gene" is a floral organ identity gene comprising a MADS box, having an *a* function in the ABC model, such as the *Arabidopsis* gene *APETALA 1* or a homologous gene from *Brassicaceae* plants.

In one embodiment of the invention, the promoter of an A-type floral organ identity gene may be a promoter derived from the *APETALA1* gene of *Arabidopsis thaliana*, such as a promoter comprising the nucleotide sequence of SEQ ID No 3 or the nucleotide sequence of SEQ ID No 4.

Alternatively, a promoter which is expressed selectively or exclusively in petals can also be used to achieve the phenotypic expression of a floral organ identity gene of the B-type selectively or exclusively in the whorl of petals (whorl 2). One example of a promoter of the latter type is a modified promoter of the *APETALA3* gene of *Arabidopsis thaliana*, which is expressed selectively in the whorl of petals of oilseed rape plants. An example of such a modified promoter would be a promoter comprising the sequence SEQ ID No 5 wherein the stamen-specific region located between the nucleotide at position 198 and the nucleotide at position 461 has been inactivated, by deletion, insertion or substitution of a number of consecutive nucleotides. It is thought that deleting or substituting at least 10 consecutive nucleotides, or at least 20 consecutive nucleotides, or at least 50 or 100 consecutive nucleotides is sufficient for inactivating the stamen-specific region of a promoter containing such a nucleotide sequence. It is also clear that the complete nucleotide sequence of SEQ ID 5 between the nucleotide at position 198 and the nucleotide at position 461 may be deleted to obtain a promoter which drives transcription of the downstream nucleotide sequence selectively or exclusively in petals of Brassicaceae plants.

The promoter may be further modified, e.g. by including into the promoter sequence at least one enhancer region, such as the enhancer region having the nucleotide sequence located between the nucleotide at position 1 to the nucleotide at position 152 of the nucleotide sequence of SEQ ID 5. This enhancer region may be repeated several times, up to at least 10 times.

The petal specific promoter may also be combined with a promoter of an A-type floral organ identity gene such as a promoter derived from the *APETALA1* gene of *Arabidopsis thaliana*.

Thus, in one embodiment of the invention, the B-type floral organ identity specific chimeric gene has a promoter region which comprises multiple repeated copies (e.g. 3 copies) of the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 1 to the nucleotide at position 152, followed by or otherwise operably linked to a region comprising the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 1 to the nucleotide at position 197, followed by or otherwise operably linked to a DNA region comprising the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 462 to the nucleotide at position 751, with the provisio that the sequence of SEQ ID No 5 from the nucleotide at position 198 to the nucleotide at position 461 is not included.

In another embodiment of the invention the B-type floral organ identity specific chimeric gene has a promoter region which comprises the immediately above mentioned nucleotide sequences, followed by or otherwise operably linked to the nucleotide sequence of SEQ ID No 3 or SEQ ID No 4.

In yet another embodiment of the invention the B-type floral organ identity specific chimeric gene has a promoter region which comprises the nucleotide sequence of SEQ ID 5 from the nucleotide at position 1 to the nucleotide at position 197 and the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 462 to the nucleotide at position 751 with the provisio that that the sequence of SEQ ID No 5 from the nucleotide at position 198 to the nucleotide at position 461 is not included, operably linked to the nucleotide sequence of SEQ ID No. 3 or 4.

It is clear that variant promoters may be used which have essentially the same biological properties, in particular direct transcription of the operably linked DNA fragment in the same manner, but differ from the mentioned promoters in nucleotide sequence by deletion, substitution or insertion of nucleotides. It should also be clear that promoters of APETALA1-like genes having essentially the same biological properties as the described promoters but which could be obtained from different plant species (such as *Brassica spp* (comprising the sequence disclosed in databases under the accession number AF126727), or *Oryza sativa*) may be used.

In an alternative embodiment of the invention, the expression of B-type floral organ identity genes may be influenced indirectly by reducing the expression of *SEPALATTA3* selectively or exclusively in the whorl 2 of the flowers. B-type floral organ identity specific chimeric genes suitable for this purpose may comprise the following DNA elements:

a) a promoter expressed selectively or exclusively in whorl 2, such as but not limited to the modified *APETALA3* promoters described elsewhere in this application;

b) a sense or antisense region, or a complementary sense and antisense region, which comprises a part of a *SEPALATTA3* gene sequence or is complementary thereto (within the ranges of sequence identity and length as described elsewhere in this application). The nucleotide sequence of the *SEPALATTA3* gene from Arabidopsis is available (previously named AGL9; Genbank Accession number AF015552), and can be used to identify *SEPALATTA*3-like genes from other *Brassicaceae* plants in a manner similar as described elsewhere in this application for the isolation or B-type genes.

It goes without saying that different B-type floral organ identity specific chimeric genes designed to reduce the phenotypic expression of different target B-type floral identity genes may be introduced simultaneously into Brassicaceae plants. The different target B-type target floral identity genes may be e.g. APETALA3 or

PISTILATTA, but may also be the different members of a multigene family of APETALA3 like or PISTILLATA like genes which can be found in some Brassicaceae plants. Equally, the different B-type floral organ identity specific chimeric genes to be combined may be any combination of anti-APETALA3, anti-PISTILLATA and anti-SEPALATTA3 chimeric genes. Such simultaneous introduction may be conveniently achieved by crossing plants each plant comprising one or more of the different B-type floral organ identity specific chimeric genes, desired to be combined, but could also be achieved by other means such as but not limited to transformation of a Brassicaceae plant comprising a certain B-type floral organ specific chimeric gene with another gene.

In another embodiment of the invention, DNA molecules comprising the B-type floral organ identity specific chimeric genes as herein described are provided.

As used herein, "a plant from the family *Brassicaceae*" is a plant which according to current botanical standards would be classified into the family *Brassicaceae* (formerly *Cruciferaeae*). *Brassicaceae* (Mustard) family members are easy to distinguish. They are annual or perennial plants with alternate leaves without stipules and simple inflorescence or branched racemes. The flowers are bilaterally symmetrical and hypogynous. With few exceptions, the flowers have 4 petals (free) alternating with 4 sepals (free); 6 stamens (4 long and 2 short);an ovary of 2 united carpels with parital placentas, 2-locular through the formation of a membranous false septum; fruit is a dehiscent capsule opening by 2 valves. *Brassicaceae* include *inter alia* the following genera : *Sisymbrium, Descurania, Alliaria, Arabidopsis, Myagrum, Isatis, Bunias, Erysimum, Hesperis, Malcolmia, Matthiola, Chorispora, Euclidium, Barbarea, Rorippa, Armoracia, Nasturtium, Dentaria, Cardamine, Cardaminopsis, Arabis, Lunaria, Alyssum, Berteroa, Lobularia, Draba, Erophila, Cochlearia, Camelina, Neslia, Capsella, Homungia, Thlaspi, Iberis, Lepidium, Cardaria, Coronopus, Subularia, Conringia, Diplotaxis, Brassica, Sinapsis, Eruca, Erucastrum, Coincya, Hirschfeldia, Cakile, Rapistrum, Crambe, Enarthrocarpus, Rhaphanus* and *Clausia*.

"Oilseed rape" as used herein, should be understood to include the species *Brassica napus*, *Brassica junceae* and *Brassica campestris*.

The obtained transformed plant can be used in a conventional breeding scheme, including cross-pollination and/or self pollination to produce more transformed plants with the same characteristics or to introduce the chimeric genes of the invention in other varieties of the same or related plant species. Seeds, including crushed seeds and seed cakes obtained from the transformed plants and containing the chimeric genes of the invention as a stable genomic insert are also encompassed by this invention.

The following non-limiting Examples describe the construction of chimeric B-type floral organ identity specific chimeric genes and the use of those nucleic acids for altering flower morphology in *Brassicaceae* . Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual,* Third Edition, Cold Spring Harbor Laboratory Press, NY, in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology, Current Protocols*, USA and in Volumes I and II of Brown (1998) *Molecular Biology LabFax*, Second Edition, Academic Press (UK). Standard materials and methods for plant molecular work are described in *Plant Molecular Biology Labfax* (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Standard materials and methods for polymerase chain reactions can be found in Dieffenbach and Dveksler (1995) *PCR Primer: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, and in McPherson at al. (2000) *PCR - Basics: From Background to Bench,* First Edition, Springer Verlag, Germany.

All the nucleotide sequences identified in this text by their database accession number are hereby incorporated by reference.

Throughout the description and Examples, reference is made to the following sequences:

SEQ ID No 1: APETALA3-like cDNA fragment from *Brassica napus*.

SEQ ID No 2: PISTILLATA-like cDNA fragment from *Brassica napus*.

SEQ ID No 3: sequence of the AP1 promoter from *Arabidopsis thaliana*.

SEQ ID No 4: sequence of the 1.1 kb fragment of the AP1 promoter from *Arabidopsis thaliana*.

SEQ ID No 5: sequence of an AP3 promoter from *Arabidopsis thaliana*.

SEQ ID No 6: oligonucleotide primer BM7

SEQ ID No 7: oligonucleotide primer BM2

SEQ ID No 8: oligonucleotide primer BM3

SEQ ID No 9: oligonucleotide primer BM6

Examples:

Example 1: Construction of B-type floral organ identity specific chimeric genes under control of a promoter of an A-type floral organ identity gene and introduction into plants.

The 3' end of the B-type floral organ identity genes from *Brassica napus*, corresponding to *APETALA3* and *PISTILLATA* from *Arabidopsis thaliana*, were amplified by PCR under standard conditions, using as template, cDNA made on total RNA isolated from flower buds from *Brassica napus* and the oligonuclotide primers BM 7 (SEQ ID No 6) and BM2 (SEQ ID No 7) for the amplification of the 3' end of *APETALA 3*-like gene (SEQ ID No 1) and oligonucleotide primers BM3 (SEQ ID No 8) and BM6 (SEQ ID No 9) for the *PISTILLATA*-like fragment (SEQ ID No ID 2).

Using standard cloning techniques, the following B-type floral organ identity specific chimeric genes have been constructed and introduced into T-DNA vectors together with appropriate selective marker gene (plant-expressible *bar* gene).

All T-DNA vectors were derived from pADP68

pTBM4 is a T-DNA vector comprising between the T-DNA borders

1) a B-type floral organ identity specific chimeric gene comprising the following operably linked DNA fragments

- **PAP1:** 1.1 kb promoter fragment that drives the expression of the *APETALA*1 gene from *Arabidopsis thaliana* (Gustafson-Brown *et al.*, 1994) having the nucleotide sequence of SEQ ID No 4;
- **BAP3**: about 380 bp of the 3'-region of the *APETALA*3 (AP3)-like MADS-box gene from *B. napus* having the sequence of SEQ ID No 1;

- **GUS**: about 250 bp EcoRV fragment of the beta- glucuronidase (*uid*A) gene from *E. coli* as spacer;
- **BAP3**: about 380 bp of the 3′-region of the *APETALA*3 (AP3)-like MADS-box gene from *B.napus* in reverse orientation having the complement of the nucleotide sequence of SEQ ID No 1;
- **3'35S**: A 213 nt fragment containing the 3'end formation signals obtained from the Cauliflower mosaic virus (Sanfacon *et al.*, 1991)

2) a chimeric *bar* gene comprising the following operably linked DNA fragments

- **PSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers et al. (1988), Plant Molec Biol.11: 745-759).
- **bar**: The coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson et al., 1987)
- **3′g7**: A 211bp DNA fragment containing the 3′ end formation signals obtained from the 3′ untranslated region of the TL-DNA gene 7 on pTIB6S3 (Dhaese et al., 1983; Velten and Schell, 1985)

pTBM6 is a T-DNA vector comprising between the T-DNA borders

1) a B-type floral organ identity specific chimeric gene comprising the following operably linked DNA fragments

- **PAP1:** 1.8 kb promoter fragment that drives the expression of the *APETALA*1 gene from *Arabidopsis thaliana* (Gustafson-Brown *et al.*, 1994) having the nucleotide sequence of SEQ ID No 3;
- **BAP3**: about 380 bp of the 3′-region of the *APETALA*3 (AP3)-like MADS-box gene from *B. napus* having the sequence of SEQ ID No 1;
- **GUS**: about 250 bp *Eco*RV fragment of the beta- glucuronidase (*uid*A) gene from *E. coli* as spacer;

- **BAP3**: about 380 bp of the 3´-region of the *APETALA*3 (AP3)-like MADS-box gene from *B.napus* in reverse orientation having the complement of the nucleotide sequence of SEQ ID No 1;
- **3'35S**: A 213 nt fragment containing the 3'end formation signals obtained from the Cauliflower mosaic virus (Sanfacon *et al.*, 1991)

2) a chimeric *bar* gene comprising the following operably linked DNA fragments

- **PSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers et al. (1988), Plant Molec Biol.11: 745-759).
- **bar**: The coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson et al., 1987)
- **3´g7**: A 211bp DNA fragment containing the 3´ end formation signals obtained from the 3´ untranslated region of the TL-DNA gene 7 on pTIB6S3 (Dhaese et al., 1983; Velten and Schell, 1985).

PTBM7 is a T-DNA vector comprising between the T-DNA borders

1) a B-type floral organ identity specific chimeric gene comprising the following operably linked DNA fragments

- **PAP1:** 1.8 kb promoter fragment that drives the expression of the *APETALA1* gene from *Arabidopsis thaliana* (Gustafson-Brown *et al.*, 1994) having the nucleotide sequence of SEQ ID No 3;
- **PISTILLATA**: about 250 bp of the 3´-region of the *PISTILLATA*-like MADS-box gene from *B. napus* having the sequence of SEQ ID No 2;
- **GUS**: about 230 bp *Nci*I fragment of the beta- glucuronidase (*uid*A) gene from *E. coli* containing the portable second intron (IV2) of the Potato light inducible tissue-specific ST-LS1 gene (Vancanneyt et al. 1990) as spacer;

- **PISTILLATA**: about 380 bp of the 3′-region of the *PISTILLATA*-like MADS-box gene from *B.napus* in reverse orientation having the complement of the nucleotide sequence of SEQ ID No 2;
- **3'35S**: A 213 nt fragment containing the 3'end formation signals obtained from the Cauliflower mosaic virus (Sanfacon *et al.*, 1991)

2) a chimeric *bar* gene comprising the following operably linked DNA fragments

- **PSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers et al. (1988), Plant Molec Biol.11: 745-759).
- **bar**: The coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson et al., 1987)
- **3′g7**: A 211bp DNA fragment containing the 3′ end formation signals obtained from the 3′ untranslated region of the TL-DNA gene 7 on pTIB6S3 (Dhaese et al., 1983; Velten and Schell, 1985).

PTBM8 is a T-DNA vector comprising between the T-DNA borders

1) a B-type floral organ identity specific chimeric gene comprising the following operably linked DNA fragments

- **PAP1:** 1.1 kb promoter fragment that drives the expression of the *APETALA1* gene from *Arabidopsis thaliana* (Gustafson-Brown *et al.*, 1994) having the nucleotide sequence of SEQ ID No 3;
- **PISTILLATA**: about 250 bp of the 3′-region of the *PISTILLATA*-like MADS-box gene from *B. napus* having the sequence of SEQ ID No 2;
- **GUS**: about 230 bp *Nci*I fragment of the beta- glucuronidase (*uid*A) gene from *E. coli* containing the portable second intron (IV2) of the Potato light inducible tissue-specific ST-LS1 gene (Vancanneyt et al. 1990) as spacer;

- **PISTILLATA**: about 380 bp of the 3'-region of the *PISTILLATA*-like MADS-box gene from *B.napus* in reverse orientation having the complement of the nucleotide sequence of SEQ ID No 2;
- **3'35S**: A 213 nt fragment containing the 3'end formation signals obtained from the Cauliflower mosaic virus (Sanfacon *et al.*, 1991)

2) a chimeric *bar* gene comprising the following operably linked DNA fragments

- **PSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers et al. (1988), Plant Molec Biol.11: 745-759).
- **bar**: The coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson et al., 1987)
- **3'g7**: A 211bp DNA fragment containing the 3' end formation signals obtained from the 3' untranslated region of the TL-DNA gene 7 on pTIB6S3 (Dhaese et al., 1983; Velten and Schell, 1985).

The T-DNA vectors were introduced into *Agrobacterium tumefaciens* C58C1Rif (pGV4000), and the resulting *Agrobacterium* strains were used to transform *Arabidopsis* plants according to the root explant inoculation method (Valvekens et al.,1988) or *Brassica napus* plants according to the hypocotyl explant inoculation method (essentially as described in De Block et al. 1989 or in WO 00/04173)

Example 2: Analysis of the phenotype of flowers of transgenic Arabidopsis and Brassica napus plants.

Different transgenic *Arabidopsis* lines comprising each of the chimeric constructs of Example 1 were analyzed for alteration of the phenotype of the flowers. For all constructs, transgenic lines were identified having double sepals and normal

stamens. In addition, flowers with short petals and normal stamen could be observed, as well as flowers with double sepals but also aberrant stamens (see Table 1)

Table 1. Phenotypic analysis of flowers of *Arabidopsis* transgenic plants

Construct	Total number of transgenic lines	Wild-type phenotype flowers	Double sepals normal stamen	Double sepals Aberrant stamen	Short petals normal stamen	Short petals carpeloid stamen
PTBM4	49	18	9	14	6	2
PTBM5	63	42	3	10	5	3
PTBM8	24	18	2	-	4	-
PTMB7	13	6	1	2	4	-

Different transgenic *Brassica napus* lines comprising each of the chimeric constructs of Example 1 or combinations thereof are analyzed for alteration of the phenotype of the flowers. Similar phenotypes as for transgenic *Arabidopsis* plants are observed.

Example 3: Construction of B-type floral organ identity specific chimeric genes under control of a promoter of APETALA3 from *A. thaliana* and introduction into *Brassica napus* plants.

Using standard cloning techniques, the following B-type floral organ identity specific chimeric genes have been constructed and introduced into T-DNA vectors together with appropriate selective marker gene (plant-expressible *bar* gene).

All T-DNA vectors were derived from pADP68

pTBM10 is a T-DNA vector comprising between the T-DNA borders

1) a B-type floral organ identity specific chimeric gene comprising the following operably linked DNA fragments

- **PAP3m3:** modified promoter of the APETALA3 gene from *Arabidopsis thaliana* operably linked to the promoter of the APETALA1 gene from *Arabidopsis thaliana* prcomprising the following operably nucleotide sequences:
  - the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 1 to the nucleotide at position 197,followed by
  - the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 462 to the nucleotide at position 751, such that the sequence of SEQ ID No 5 from the nucleotide at position 198 to the nucleotide at position 461 is not included
  - the nucleotide sequence of SEQ ID No 3 (1.1 kb fragment of the APETALA1 promoter).

- **PISTILLATA**: about 250 bp of the 3´-region of the *PISTILLATA*-like MADS-box gene from *B. napus* having the sequence of SEQ ID No 2;
- **GUS**: about 230 bp *Nci*I fragment of the beta- glucuronidase (*uid*A) gene from *E. coli* containing the portable second intron (IV2) of the Potato light inducible tissue-specific ST-LS1 gene (Vancanneyt et al. 1990) as spacer;
- **PISTILLATA**: about 380 bp of the 3´-region of the *PISTILLATA*-like MADS-box gene from *B.napus* in reverse orientation having the complement of the nucleotide sequence of SEQ ID No 2;
- **3'35S**: A 213 nt fragment containing the 3'end formation signals obtained from the Cauliflower mosaic virus (Sanfacon *et al.*, 1991)

2) a chimeric *bar* gene comprising the following operably linked DNA fragments

- **PSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers et al. (1988), Plant Molec Biol.11: 745-759).
- **bar**: The coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson et al., 1987)
- **3'g7**: A 211bp DNA fragment containing the 3' end formation signals obtained from the 3' untranslated region of the TL-DNA gene 7 on pTIB6S3 (Dhaese et al., 1983; Velten and Schell, 1985).

pTBM11 is a T-DNA vector comprising between the T-DNA borders

1) a B-type floral organ identity specific chimeric gene comprising the following operably linked DNA fragments

- **PAP3m2:** modified promoter of the APETALA3 gene from *Arabidopsis thaliana* comprising the following operably nucleotide sequences:
  - 3 copies of the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 1 to the nucleotide at position 152 in direct repeat followed by
  - the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 1 to the nucleotide at position 197,followed by
  - the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 462 to the nucleotide at position 751, such that the sequence of SEQ ID No 5 from the nucleotide at position 198 to the nucleotide at position 461 is not included

- **PISTILLATA**: about 250 bp of the 3'-region of the *PISTILLATA*-like MADS-box gene from *B. napus* having the sequence of SEQ ID No 2;
- **GUS**: about 230 bp *Nci*I fragment of the beta- glucuronidase (*uid*A) gene from *E. coli* containing the portable second intron (IV2) of the Potato light inducible tissue-specific ST-LS1 gene (Vancanneyt et al. 1990) as spacer;

- **PISTILLATA**: about 380 bp of the 3´-region of the *PISTILLATA*-like MADS-box gene from *B.napus* in reverse orientation having the complement of the nucleotide sequence of SEQ ID No 2;
- **3'35S**: A 213 nt fragment containing the 3'end formation signals obtained from the Cauliflower mosaic virus (Sanfacon *et al.*, 1991)

2) a chimeric *bar* gene comprising the following operably linked DNA fragments

- **PSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers et al. (1988), Plant Molec Biol.11: 745-759).
- **bar**: The coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson et al., 1987)

3´g7: A 211bp DNA fragment containing the 3´ end formation signals obtained from the 3´ untranslated region of the TL-DNA gene 7 on pTIB6S3 (Dhaese et al., 1983; Velten and Schell, 1985).

pTBM12 is a T-DNA vector comprising between the T-DNA borders

1) a B-type floral organ identity specific chimeric gene comprising the following operably linked DNA fragments

- **PAP3m:** modified promoter of the APETALA3 gene from *Arabidopsis thaliana* comprising the following operably nucleotide sequences:
  - 3 copies of the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 1 to the nucleotide at position 152 in direct repeat followed by
  - the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 1 to the nucleotide at position 197,followed by
  - the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 462 to the nucleotide at position 751, such that the sequence of SEQ ID No 5 from the nucleotide at position 198 to the nucleotide at position 461 is not included
  - the nucleotide sequence of SEQ ID No 3 (1.1 kb fragment of the APETALA1 promoter).

- **PISTILLATA**: about 250 bp of the 3′-region of the *PISTILLATA*-like MADS-box gene from *B. napus* having the sequence of SEQ ID No 2;
- **GUS**: about 230 bp *Nci*I fragment of the beta- glucuronidase (*uid*A) gene from *E. coli* containing the portable second intron (IV2) of the Potato light inducible tissue-specific ST-LS1 gene (Vancanneyt et al. 1990) as spacer;
- **PISTILLATA**: about 380 bp of the 3′-region of the *PISTILLATA*-like MADS-box gene from *B.napus* in reverse orientation having the complement of the nucleotide sequence of SEQ ID No 2;
- **3'35S**: A 213 nt fragment containing the 3'end formation signals obtained from the Cauliflower mosaic virus (Sanfacon *et al.*, 1991)

2) a chimeric *bar* gene comprising the following operably linked DNA fragments

- **PSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers et al. (1988), Plant Molec Biol.11: 745-759).
- **bar**: The coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson et al., 1987)
- **3′g7**: A 211bp DNA fragment containing the 3′ end formation signals obtained from the 3′ untranslated region of the TL-DNA gene 7 on pTIB6S3 (Dhaese et al., 1983; Velten and Schell, 1985).

pTBM13 is a T-DNA vector comprising between the T-DNA borders

1) a B-type floral organ identity specific chimeric gene comprising the following operably linked DNA fragments

- **PAP3m:** modified promoter of the APETALA3 gene from *Arabidopsis thaliana* operably linked to the APETALA1 promoter comprising the following operably nucleotide sequences:
  - 3 copies of the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 1 to the nucleotide at position 152 in direct repeat followed by

- the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 1 to the nucleotide at position 197,followed by
- the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 462 to the nucleotide at position 751, such that the sequence of SEQ ID No 5 from the nucleotide at position 198 to the nucleotide at position 461 is not included
- the nucleotide sequence of SEQ ID No 3 (1.1 kb fragment of the APETALA1 promoter).

- **BAP3**: about 380 bp of the 3'-region of the *APETALA*3 (AP3)-like MADS-box gene from *B. napus* having the sequence of SEQ ID No 1;
- **GUS**: about 250 bp *Eco*RV fragment of the beta- glucuronidase (*uid*A) gene from *E. coli* as spacer;
- **BAP3**: about 380 bp of the 3'-region of the *APETALA*3 (AP3)-like MADS-box gene from *B.napus* in reverse orientation having the complement of the nucleotide sequence of SEQ ID No 1;
- **3'35S**: A 213 nt fragment containing the 3'end formation signals obtained from the Cauliflower mosaic virus (Sanfacon *et al.*, 1991)

2) a chimeric *bar* gene comprising the following operably linked DNA fragments

- **PSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers et al. (1988), Plant Molec Biol.11: 745-759).
- **bar**: The coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson et al., 1987)
- **3'g7**: A 211bp DNA fragment containing the 3' end formation signals obtained from the 3' untranslated region of the TL-DNA gene 7 on pTIB6S3 (Dhaese et al., 1983; Velten and Schell, 1985).

pTBM14 is a T-DNA vector comprising between the T-DNA borders

1) a B-type floral organ identity specific chimeric gene comprising the following operably linked DNA fragments

- **PAP3m2:** modified promoter of the APETALA3 gene from *Arabidopsis thaliana* comprising the following operably nucleotide sequences:
  - 3 copies of the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 1 to the nucleotide at position 152 in direct repeat followed by
  - the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 1 to the nucleotide at position 197,followed by
  - the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 462 to the nucleotide at position 751, such that the sequence of SEQ ID No 5 from the nucleotide at position 198 to the nucleotide at position 461 is not included;
- **BAP3**: about 380 bp of the 3´-region of the *APETALA*3 (AP3)-like MADS-box gene from *B. napus* having the sequence of SEQ ID No 1;
- **GUS**: about 250 bp *Eco*RV fragment of the beta- glucuronidase (*uid*A) gene from *E. coli* as spacer;
- **BAP3**: about 380 bp of the 3´-region of the *APETALA*3 (AP3)-like MADS-box gene from *B.napus* in reverse orientation having the complement of the nucleotide sequence of SEQ ID No 1;
- **3'35S**: A 213 nt fragment containing the 3'end formation signals obtained from the Cauliflower mosaic virus (Sanfacon *et al.*, 1991)

2) a chimeric *bar* gene comprising the following operably linked DNA fragments

- **PSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers et al. (1988), Plant Molec Biol.11: 745-759).
- **bar**: The coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson et al., 1987)

- **3'g7**: A 211bp DNA fragment containing the 3' end formation signals obtained from the 3' untranslated region of the TL-DNA gene 7 on pTIB6S3 (Dhaese et al., 1983; Velten and Schell, 1985).

pTBM15 is a T-DNA vector comprising between the T-DNA borders

1) a B-type floral organ identity specific chimeric gene comprising the following operably linked DNA fragments

- **PAP3m3:** modified promoter of the APETALA3 gene from *Arabidopsis thaliana* operably linked to the promoter of the APETALA1 gene from *Arabidopsis thaliana* prcomprising the following operably nucleotide sequences:
  - the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 1 to the nucleotide at position 197,followed by
  - the nucleotide sequence of SEQ ID No. 5 from the nucleotide at position 462 to the nucleotide at position 751, such that the sequence of SEQ ID No 5 from the nucleotide at position 198 to the nucleotide at position 461 is not included, followed by
  - the nucleotide sequence of SEQ ID No 3 (1.1 kb fragment of the APETALA1 promoter).
- **BAP3**: about 380 bp of the 3'-region of the *APETALA*3 (AP3)-like MADS-box gene from *B. napus* having the sequence of SEQ ID No 1;
- **GUS**: about 250 bp *Eco*RV fragment of the beta- glucuronidase (*uid*A) gene from *E. coli* as spacer;
- **BAP3**: about 380 bp of the 3'-region of the *APETALA*3 (AP3)-like MADS-box gene from *B.napus* in reverse orientation having the complement of the nucleotide sequence of SEQ ID No 1;
- **3'35S**: A 213 nt fragment containing the 3'end formation signals obtained from the Cauliflower mosaic virus (Sanfacon *et al.*, 1991)

2) a chimeric *bar* gene comprising the following operably linked DNA fragments

- **PSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers et al. (1988), Plant Molec Biol.11: 745-759).
- **bar**: The coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson et al., 1987)
- **3'g7**: A 211bp DNA fragment containing the 3' end formation signals obtained from the 3' untranslated region of the TL-DNA gene 7 on pTIB6S3 (Dhaese et al., 1983; Velten and Schell, 1985).

The T-DNA vectors are introduced into *Agrobacterium tumefaciens* C58C1Rif (pGV4000), and the resulting Agrobacterium strains are used to transform *Brassica napus* plants according to the hypocotyl explant inoculation method (essentially as described in De Block et al. 1989 or in WO 00/04173).

Different transgenic *Brassica napus* lines comprising each of the chimeric constructs of Example 3 are analyzed for alteration of the phenotype of the flowers. Flowers with double sepals or sepaloids and normal stamens are observed for a number of the transgenic lines.

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Claims

1. A method for obtaining a plant from the family Brassicaceae with altered floral morphology comprising the steps of
   a. introducing into a cell of said plant a B-type floral organ identity specific chimeric gene to generate a transgenic cell; and
   b. regenerating a transgenic plant from said transgenic cell;

characterized in that said B-type floral organ identity specific chimeric gene is capable of reducing or inhibiting the phenotypic expression of a target B-type floral organ identity gene selectively in the whorl 2 of a flower or flower primordia of said transgenic plants and that said flowers of said transgenic plant have

   i) an additional whorl of sepals or sepaloids;
   ii) no whorl of petals; and
   iii) a whorl of functional stamens

when compared to flowers of normal untransformed plants.

2. The method of claim 1, wherein said B-type floral organ identity specific gene comprises a first nucleotide sequence having at least 70% sequence identity to a part of at least 25 consecutive nucleotides of the nucleotide sequence of a target B-type floral organ identity gene of said plant; or comprising a first nucleotide sequence having at least 70% sequence identity to the complement of a part of at least 25 consecutive nucleotides of the nucleotide sequence of a target B-type floral organ identity gene of said plant with the provisio that said nucleotide sequence of said target B-type floral organ identity gene does not comprise the MADS box encoding nucleotide sequence.

3. The method of claim 2, wherein said target B-type floral organ identity gene is selected from the group of APETALA3, PISTILLATA, a gene homologous to APETALA3 and a gene homologous to PISTILLATA.

4. The method of claim 2 or claim 3, wherein said first nucleotide sequence has at least 70% sequence identity to a nucleotide sequence of at least 25 consecutive nucleotides selected from the group consisting of the nucleotide sequence of SEQ ID No 1, the nucleotide sequence of SEQ ID No 2, the complement of the nucleotide sequence of SEQ ID 1 and the complement of the nucleotide sequence of SEQ ID 2.

5. The method of any one of claims 2 to 4, wherein said B-type floral organ identity specific chimeric gene comprises a second nucleotide sequence having at least 70% sequence identity to a part of at least 25 consecutive nucleotides of the nucleotide sequence of a target B-type floral organ identity gene of said plant or complementary to said nucleotide sequence of said target B-type floral organ identity gene of said plant, and wherein the RNA molecule generated upon transcription of said B-type floral organ identity gene-silencing chimeric gene is capable of forming a double-stranded region between the regions of said transcribed RNA molecule transcribed from said first and second nucleotide sequence.

6. The method of claim 5, wherein said B-type floral organ identity specific chimeric gene comprises a spacer region between said first and said second nucleotide sequence.

7. The method of claim 6, wherein said spacer region comprises an intron.

8. The method of claim 5, wherein said first nucleotide sequence comprises the nucleotide sequence of SEQ ID No 1 and said second nucleotide sequence comprises the complement of SEQ ID No 1.

9. The method of claim 5, wherein said first nucleotide sequence comprises the nucleotide sequence of SEQ ID No 2 and said second nucleotide sequence comprises the complement of SEQ ID No 2.

10. The method of any one of claims 1 to 9, wherein said B-type floral organ identity specific chimeric gene comprises a promoter of an A-type floral organ identity gene.

11. The method of claim 10, wherein said promoter of an A-type floral organ identity gene is a promoter from APETALA1 or a gene homologous to APETALA1.

12. The method of claim 10, wherein said promoter of an A-type floral organ identity gene comprises the nucleotide sequence of SEQ ID No 3.

13. The method of claim 10, wherein said promoter of an A-type floral organ identity gene comprises the nucleotide sequence of SEQ ID No 4.

14. The method of any one of claims 1 to 9, wherein said B-type floral organ identity specific chimeric gene comprises a promoter directing transcription of said B-type floral organ identity specific chimeric gene selectively in whorl 2 of flowers or flower primordia when introduced into cells of plants of the genus Brassica.

15. The method of claim 14, wherein said promoter is a modified promoter from APETALA 3 gene from Arabidopsis thaliana.

16. The method of claim 15, wherein said promoter comprises the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 1 to the nucleotide at position 152 and the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 462 to the nucleotide position at position 751 with the provisio that said promoter does not comprise the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 198 to the nucleotide at position 461.

17. The method of any one of claims 1 to 16, wherein said plant is a plant from the genus Brassica or a closely related genus.

18. The method of claim 17, wherein said plant is oilseed rape.

19. The method of any one of claims 1 to 18, further comprising the step of
c. obtaining progeny plants from said transgenic plant.

20. A DNA molecule comprising a B-type floral organ identity specific chimeric gene, characterized in that upon introduction into the genome of a plant of the family Brassicaceae of said B-type floral organ identity specific chimeric gene, said B-type floral organ identity specific chimeric gene is capable of reducing or inhibiting the phenotypic expression of a target B-type floral organ identity gene selectively in whorl 2 of a flower or flower primordia and said flowers of said transgenic plant have
i) an additional whorl of sepals or sepaloids;
ii) no whorl of petals; and
iii) a functional whorl of stamens
when compared to flowers of normal untransformed plants.

21. The DNA molecule of claim 20, wherein said B-type floral organ identity specific gene comprises a first nucleotide sequence having at least 70% sequence identity to a part of at least 25 consecutive nucleotides of the nucleotide sequence of a target B-type floral organ identity gene of said plant; or comprising a first nucleotide sequence having at least 70% sequence identity to the complement of a part of at least 25 consecutive nucleotides of the nucleotide sequence of a target B-type floral organ identity gene of said plant with the provisio that said nucleotide sequence of said target B-type floral organ identity gene does not comprise the MADS box encoding nucleotide sequence.

22. The DNA molecule of claim 21, wherein said target B-type floral organ identity gene is selected from the group of APETALA3, PISTILLATA, a gene homologous to APETALA3 and a gene homologous to PISTILLATA.

23. The DNA molecule of claim 21 or claim 22, wherein said first nucleotide sequence has at least 70% sequence identity to a nucleotide sequence of at least 25 consecutive nucleotides selected from the group consisting of the nucleotide sequence of SEQ ID No 1, the nucleotide sequence of SEQ ID No 2, the complement of SEQ ID No 1 and the complement of SEQ ID No 2.

24. The DNA molecule of any one of claims 20 to 23, wherein said B-type floral organ identity specific chimeric gene comprises a second nucleotide sequence having at least 70% sequence identity to a part of at least 25 consecutive nucleotides of the nucleotide sequence of a target B-type floral organ identity gene of said plant or complementary to said nucleotide sequence of said target B-type floral organ identity gene of said plant, and wherein the RNA molecule generated upon transcription of said B-type floral organ identity gene-silencing chimeric gene is capable of forming a double-stranded region between the regions of said transcribed RNA molecule transcribed from said first and second nucleotide sequence.

25. The DNA molecule of claim 24, wherein said B-type floral organ identity specific chimeric gene comprises a spacer region between said first and said second nucleotide sequence.

26. The DNA molecule of claim 25, wherein said spacer region comprises an intron.

27. The DNA molecule of claim 24, wherein said first nucleotide sequence comprises the nucleotide sequence of SEQ ID No 1 and said second

nucleotide sequence comprises the complement of nucleotide sequence of SEQ ID No 1.

28. The DNA molecule of claim 24, wherein said first nucleotide sequence comprises the nucleotide sequence of SEQ ID No 2 and said second nucleotide sequence comprises the complement of nucleotide sequence of SEQ ID No 2.

29. The DNA molecule of any one of claims 20 to 28 wherein said B-type floral organ identity specific chimeric gene comprises a promoter of an A-type floral organ identity gene.

30. The DNA molecule of claim 29, wherein said promoter of an A-type floral organ identity gene is a promoter from APETALA1 or a gene homologous to APETALA1.

31. The DNA molecule of claim 30, wherein said promoter of an A-type floral organ identity gene comprises the nucleotide sequence of SEQ ID No 3.

32. The DNA molecule of claim 30, wherein said promoter of an A-type floral organ identity gene comprises the nucleotide sequence of SEQ ID No 4.

33. The DNA molecule of any one of claims 20 to 28, wherein said B-type floral organ identity specific chimeric gene comprises a promoter directing transcription of said B-type floral organ identity specific chimeric gene selectively in whorl 2 of flowers or flower primordia when introduced into cells of plants of the genus Brassica.

34. The DNA molecule of claim 33, wherein said promoter is a modified promoter from APETALA 3 gene from Arabidopsis.

35. The DNA molecule of claim 35, wherein said promoter comprises the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 1 to the nucleotide at position 152 and the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 462 to the nucleotide position at position 751 with the provisio that said promoter does not comprise the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 198 to the nucleotide at position 461.

36. A plant from the family Brassicaceae comprising a DNA molecule of any one of claims 20 to 35 integrated into the genome of the cells of said plant.

37. The plant of claim 36, wherein said plant is a plant from the genus Brassica or a closely related genus.

38. The plant of claim 37, wherein said plant is oilseed rape.

39. A seed of the plant of any one of claims 36 to 38, comprising a DNA molecule of any one of claims 20 to 35.

40. The seed of claim 39, which is a crushed seed.

41. Use of a DNA molecule according to any one of claims 20 to 35 to obtain a plant of the family Brassicaceae with altered flowers, said flowers having

i) an additional whorl of sepals or sepaloids;

ii) no whorl of petals; and

iii) a functional whorl of stamens

when compared to flowers of normal untransformed plants.

SEQUENCE LISTING

<110> Aventis CropScience N.V.
De Block, Marc
Byzova, Marina

<120> Brassicaceae with altered floral development

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<151> 2000-10-03

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Oligonucleotide primer BM7

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